

A comparative study on the efficacy of a pest-specific and prey-marking enzyme-linked immunosorbent assay for detection of predation

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Abstract

The efficacy of two different antigen–antibody combinations to detect predation on eggs of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) was compared. The first method was an indirect enzyme-linked immunosorbent assay (ELISA) using monoclonal antibody-based gut content analysis that detects *H. armigera* egg protein. The second method was a sandwich ELISA that detects an exotic protein [rabbit immunoglobulin G (IgG)] applied as an external marker to *H. armigera* eggs. The target predators were the predatory beetles *Dicranolaius bellulus* (Guerin-Meneville) (Coleoptera: Melyridae) and *Hippodamia variegata* (Goeze) (Coleoptera: Coccinellidae). Beetles were fed with *H. armigera* eggs that had been marked with rabbit IgG and then held at various intervals after prey consumption. Each individual beetle was then assayed by both ELISA techniques to identify the prey remains in their guts. The two ELISA methods were further tested on field-collected predators. Specifically, protein-marked egg masses were strategically placed in a cotton field. Then, predators from surrounding cotton plants were collected at various time intervals after the marked eggs were exposed and assayed by both ELISAs to detect the frequency of predation on the marked eggs. The rabbit IgG-specific sandwich ELISA had a higher detection rate than the *H. armigera*-specific indirect ELISA under controlled and field conditions for both predator species. Moreover, a greater proportion of field-collected *D. bellulus* tested positive for predation than *H. variegata*. The advantages and disadvantages of using prey-marking ELISAs instead of pest-specific ELISA assays are discussed.

Introduction

Insect predation is difficult to quantify under natural conditions, because predators and prey are often cryptic, secretive, or nocturnal (Hagler & Cohen, 1990). Unless observed directly in the act of feeding upon a prey item, a predator leaves little or no trace of its feeding activity. The development of immunological and DNA-based techniques to detect prey in the gut contents of individual predators is a significant advance in the assessment of predation, because these methods provide indirect evidence of

predatory events (reviewed by Greenstone, 1996; Hagler & Naranjo, 1996; Sheppard & Harwood, 2005).

Two immunological tools have proved useful for detecting predation when used in enzyme-linked immunosorbent assays (ELISA): insect-specific antigens detected by monoclonal antibodies (MAb) (Greenstone & Morgan, 1989; Hagler et al., 1992; Symondson et al., 1999; Harwood et al., 2005; Fournier et al., 2006) and exotic antigens (proteins) that are applied to target prey externally and/or internally (Hagler & Durand, 1994; Hagler, 2006). The most appropriate method should be chosen only after tests conducted in the habitat of interest using the target predator and prey species. Ideally, the ELISA method chosen will maximize the likelihood of prey detection for that particular predator–prey complex. With the development of multiple mark sandwich ELISAs, it is now possible to

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test simultaneously for different prey items within an individual predator without the burden of having to develop one or more prey-specific MAbs (Hagler, 2006). Also, the type of ELISA used (indirect or sandwich) affects the sensitivity of target-specific assays (Hagler, 1998; Fournier et al., 2006). For gut content analyses, the sandwich ELISA is the more sensitive assay. Unfortunately, most of the prey-specific ELISAs developed to date use the indirect ELISA format (see Hagler, 1998; Sheppard & Harwood, 2005). Other factors that affect the antigen half-life (whether target-specific or exotic) include digestion time, temperature, meal size, predator species, and physiological status (e.g., age) of the prey (Hagler et al., 1992; Greenstone & Hunt, 1993; Symondson & Liddell, 1993; Hagler & Naranjo, 1997; Agusti et al., 1999; Harwood et al., 2001). Despite these limitations, ELISA methods have given significant insight into the feeding patterns of agricultural predator complexes (e.g., Symondson et al., 2000; Harwood et al., 2004), particularly those found in North American cotton crops (Hagler & Naranjo, 1994a,b, 2004, 2005). No equivalent research exists for the arthropod predator complex that occurs within Australian cotton (described by Johnson et al., 2000).

Helicoverpa armigera (Hübner) and *Helicoverpa punctigera* (Wallengren) (Lepidoptera: Noctuidae) are the two major noctuid pests in Australian cotton. Development of insecticide resistance has been more rapid in *H. armigera* than in *H. punctigera* (Forrester et al., 1993), and there is potential for *H. armigera* to develop resistance to genetically modified cotton varieties (INGARD®, now superseded by Bollgard®, Monsanto, Melbourne, Victoria, Australia) that express insecticidal toxins from *Bacillus thuringiensis* (Bird & Akhurst 2007). The introduction of INGARD® cotton has been associated with fewer insecticide applications against *Helicoverpa* spp. and a greater abundance of beneficial arthropods (Mansfield et al., 2006). Concerns over insecticide control failures and the future sustainability of cotton production in Australia have raised industry awareness of exploiting beneficial insects to help control pest populations (Fitt, 2000; Mensah, 2002a, b). To maximize benefits from arthropod predators in Australian cotton crops, it is important to identify which predator species have the greatest impact on key pests such as *H. armigera*.

An MAb specific to *H. armigera* was developed as part of a test kit to distinguish eggs of *H. armigera* and *H. punctigera* in the field (Trowell et al., 2000). The prior availability of this pest-specific antibody provided an opportunity to develop an ELISA for indirect measurement of predation on *H. armigera* without the expense of developing a new MAb solely for the purpose of predation studies (Mansfield, 2004). Moreover, the development of a prey-marking ELISA provides an easy and cost-effective alternate method

for the indirect measurement of predation (Hagler, 2006) and facilitates the comparison of the two methods to assess predation in a novel predator–prey complex.

A native predator that can be extremely abundant in Australian cotton fields (Stanley, 1997; Mansfield et al., 2006) is the red and blue beetle, *Dicranolaius bellulus* (Guerin-Meneville) (Coleoptera: Melyridae). Adults readily feed on *Helicoverpa* spp. eggs and larvae under controlled conditions (Room, 1979; Stanley, 1997; Horne et al., 2000) and on *H. armigera* eggs in field cages (Stanley, 1997). Another potential predator of *H. armigera* is the exotic ladybird, *Hippodamia variegata* (Goeze) (Coleoptera: Coccinellidae). This beetle was first recorded in Australia in 2000 and has spread rapidly into Queensland and New South Wales (Franzmann, 2002). The only prey recorded so far for *H. variegata* in Australia are aphids and thrips (Franzmann, 2002), although it is reported to feed on noctuid eggs under controlled conditions (Araya et al., 1997).

In this study, we compare the efficacy of the anti-*H. armigera* indirect ELISA to an anti-rabbit immunoglobulin G (IgG) sandwich ELISA for detecting egg remains in the guts of these two predators. Beetles were fed with *H. armigera* eggs that had been marked with rabbit IgG under controlled conditions and then assayed by both ELISAs at increasing time intervals after prey consumption. The two ELISA methods were further tested under field conditions by placing sentinel *H. armigera* egg masses marked with rabbit IgG into a cotton crop, collecting predators from the surrounding cotton plants at various time intervals after the marked eggs were exposed, and assaying them by both ELISAs.

Materials and methods

Reactivity of the *Helicoverpa armigera*-specific and rabbit IgG-specific ELISAs

The *H. armigera* MAb-based ELISA described below and the anti-rabbit IgG ELISA described by Hagler et al. (1992) were used for this study. Each ELISA was tested for reactivity to unmarked and marked *H. armigera* eggs, to two potential *H. armigera* beetle predators (*D. bellulus* and *H. variegata*), and to eight potential alternate prey items commonly found in Australian cotton crops. Arthropods examined for the ELISA cross-reactivity tests were collected from fields located at the Australian Cotton Research Institute (ACRI, Narrabri, New South Wales, Australia) and frozen after collection at -80°C until tested. Voucher specimens of the arthropods tested have been deposited at ACRI.

Anti-*Helicoverpa armigera* indirect ELISA

Individual arthropods were ground in 500 μl of Tris-buffered saline (TBS, pH 7.5) and centrifuged at 22 000 g

for 2 min. Plates were incubated at 25 °C at all stages of the assay. A 100- μ l aliquot of each macerated arthropod was placed in an individual well of a flat-bottomed Falcon Pro-Bind 96-well assay plate (Becton-Dickinson, North Ryde, New South Wales, Australia) and incubated overnight. The next day, the sample was discarded from the plate and a 300- μ l aliquot of 1% non-fat dry milk in distilled water was added to each well for 1 h to block unoccupied antigenic sites in the wells. The non-fat milk was then discarded and 100 μ l of anti-*H. armigera* MAb (Trowell et al., 2000) undiluted supernatant was added to each well for 2 h. The MAb was discarded and the plate washed three times with TBS-Tween-20 (0.05%) and twice with TBS. Rabbit anti-mouse peroxidase-conjugated immunoglobulins (Dako, Botany, New South Wales, Australia) were diluted 1:500 in 1% non-fat milk solution and 100 μ l added to each well for 1 h. This solution was then discarded and the plate was washed as described above. Finally, 100 μ l of 2,2'-azino-di-[3-ethylbenzthiazoline-6-sulfonic acid] and hydrogen peroxide (9:1 ratio) substrate solution (HRP substrate kit; Bio-Rad, Regents Park, New South Wales, Australia) was added to each well for 2 h. The absorbance of each well was read using a Benchmark Microplate reader (Bio-Rad) set at 415 nm. Every plate contained one column (eight wells) of (i) negative controls (*H. armigera* eggs, no MAb, all other reagents as described), (ii) positive controls (*H. armigera* eggs plus MAb and all other reagents), and (iii) reagent blanks (100 μ l of TBS instead of arthropod or *H. armigera* macerate, MAb and all other reagents as described).

Anti-rabbit IgG sandwich ELISA

Arthropod samples were prepared as described above for the indirect ELISA. The sandwich ELISA is described in detail by Hagler (2006). Each assay plate was coated with 100 μ l per well of anti-rabbit IgG (product no. R2004; Sigma-Aldrich, Castle Hill, New South Wales, Australia) diluted 1:500 in deionised water and incubated overnight at 4 °C. The next day, the primary antibody was discarded and a 1% solution of non-fat dry milk in distilled water was added to each well for 30 min at 27 °C to block unoccupied antigenic sites. The milk solution was then discarded and a 100- μ l aliquot of each crushed sample was added to each well and incubated for 1 h at 27 °C. The sample was discarded and the plate was washed three times with TBS-Tween-20 (0.05%) and twice with TBS. Anti-rabbit IgG peroxidase conjugate (product no. A6154; Sigma-Aldrich) diluted 1:1000 in 1% milk was added to each well (100 μ l) and incubated for 1 h at 27 °C. This solution was then discarded and the plate washed as before. Finally, 100 μ l of HRP substrate solution was added to each well and incubated for 2 h at 27 °C. The absorbance of each well was read at

415 nm. Each plate included negative controls, positive controls, and reagent blanks similar to the indirect ELISA.

Comparative efficacy of *Helicoverpa armigera*-specific and exotic protein-specific ELISAs

Helicoverpa armigera eggs were supplied from a colony reared on artificial diet (Teakle & Jensen, 1985) and maintained by CSIRO Entomology, Narrabri, New South Wales, Australia. Adult *D. bellulus* and *H. variegata* were collected from cotton and other crops, gardens, and weeds around Narrabri, New South Wales, Australia, as needed for these experiments. Predators were kept isolated with water available but no prey for at least 24 h prior to each experiment. A subsample of individuals from each species was frozen after the isolation period to act as negative controls.

Rabbit IgG was diluted to 5.0 mg ml⁻¹ in deionised water and 1.0 ml of this solution was applied to *H. armigera* eggs attached to paper towel using a perfume atomiser (Hagler & Jackson, 1998). The marked eggs were left for 1 h to dry and then an egg mass containing ~5–10 marked *H. armigera* eggs was given to a predator in an individual Petri dish (50 mm in diameter). The predators were observed until the eggs were consumed and then frozen at -80 °C at 0, 1, 2, 4, or 24 h after feeding. Each predator, including negative control specimens (i.e., predators that were known not to contain egg in their gut), was assayed by the two ELISAs described above. The critical threshold for detection of *H. armigera* eggs within the gut contents of *D. bellulus* and *H. variegata* was set at the mean absorbance + 3 SD of the negative control beetles tested using each ELISA method (Hagler et al., 1992).

In November 2004, *H. armigera* eggs attached to paper towel were marked with rabbit IgG using a perfume atomiser as described above. Marked eggs were placed at a density of 10 m⁻¹ in a 10 row \times 10 m section of conventional cotton at the Queensland Department of Primary Industries Research Station (Biloela, Queensland, Australia). The cotton plants were approximately 0.3–0.5 m in height and had not begun to flower. Predators were collected by visual searching of 6 \times 1 m sections of cotton in the release plot at 12, 24, 36, and 48 h after egg placement. All predators were assayed by the indirect and sandwich ELISAs described above to determine the percentage of predators feeding on *H. armigera* eggs in nature.

Results

Reactivity of the *Helicoverpa armigera*-specific and rabbit IgG-specific ELISAs

As expected, the anti-*H. armigera* ELISA yielded a strong positive reaction (average absorbance >2.0; Figure 1), regardless of whether the egg was marked with rabbit IgG or not.

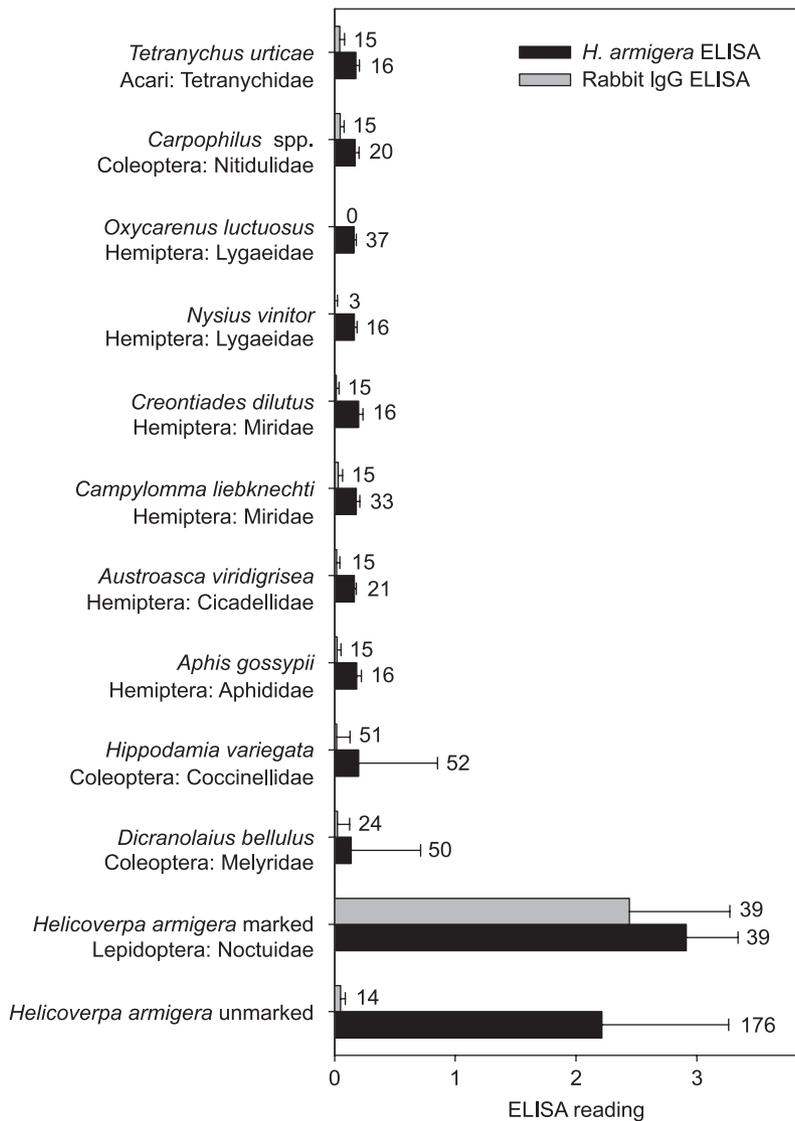


Figure 1 Mean (+ SD) enzyme-linked immunosorbent assay (ELISA) absorbance values for potential non-target prey species, the target predators (*Dicranolaius bellulus* and *Hippodamia variegata*), and the target prey [*Helicoverpa armigera* with and without rabbit immunoglobulin G (IgG) marker] assayed by the *H. armigera*-specific and rabbit IgG-specific ELISA. The numbers next to the error bars are the sample size. Non-target prey were *Tetranychus urticae* (Acari: Tetranychidae), *Carpophilus* spp. (Coleoptera: Nitidulidae), and six Hemiptera: *Oxycarenus luctuosus*, *Nysius vinitor* (Lygaeidae), *Creontiades dilutus*, *Campylomma liebknechti* (Miridae), *Austroasca viridigrisea* (Cicadellidae), and *Aphis gossypii* (Aphididae).

The cross-reactivity of the anti-*H. armigera* ELISA to the other insect species was relatively high with ELISA optical density values ranging between (mean \pm SE) 0.158 ± 0.23 for *Oxycarenus luctuosus* (Montrouzier) to 0.200 ± 0.034 for *Creontiades dilutus* (stål) (Figure 1). However, the reactivity to *H. armigera* was at least 12 times greater than the highest non-target optical density. The anti-rabbit IgG ELISA only yielded a strong positive reaction for marked *H. armigera* eggs (ELISA absorbance >2.0), but was unresponsive to unmarked eggs (ELISA absorbance <0.1) and the other insect species tested (ELISA absorbance <0.05).

***Helicoverpa armigera* antigen detection and decay rates**

The anti-rabbit IgG ELISA readily (i.e., with 100% efficiency) detected the rabbit protein-marked *H. armigera*

eggs in the guts of *D. bellulus* and *H. variegata* for 4 and 24 h after feeding, respectively (Figure 2). The anti-*H. armigera* ELISA was not nearly as effective as the anti-rabbit IgG ELISA at detecting prey in the guts of the two predators, particularly for *H. variegata* ($<50\%$ efficiency after 2 h).

Detection of predation in the field

More than 100 predators were collected from the cotton crop containing the sentinel *H. armigera* egg masses marked with rabbit IgG. Each species was pooled across replicates, because too few predators of any one species were collected to warrant separate analysis for each sampling period. In total, the anti-rabbit IgG ELISA detected marked eggs in 26% of the field-collected predators, whereas the anti-*H. armigera* ELISA only detected egg antigen in 5% of the

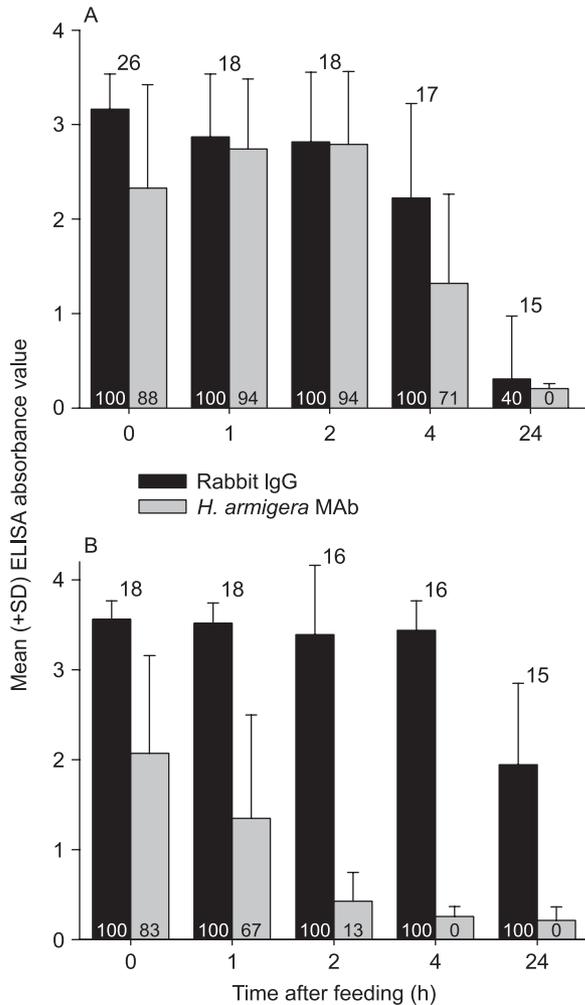


Figure 2 Mean (+ SD) absorbance values for (A) *Dicranolaius bellulus* and (B) *Hippodamia variegata* fed with *Helicoverpa armigera* eggs marked with rabbit immunoglobulin G (IgG) and then assayed with either the rabbit IgG-specific sandwich enzyme-linked immunosorbent assay (ELISA) or the *H. armigera*-specific indirect ELISA. The number of samples assayed is given above each pair of bars and the percentage of positive samples appears at the bottom of each bar.

same field-collected predators (Figures 3 and 4). Moreover, every predator that tested positive for the presence of *H. armigera* egg antigen by the anti-*H. armigera* ELISA also was tested positive by the anti-rabbit IgG ELISA (obviously, the opposite was not true). Predation by *D. bellulus* and *H. variegata* was detected more frequently with the rabbit IgG-specific ELISA than the *H. armigera*-specific ELISA (Figures 3 and 4A,B). Two other predators frequently encountered in the field, the transverse ladybird, *Coccinella transversalis* Fabricius, and the nightstalker

spider, *Cheiracanthium* spec. ($n > 10$), were also tested by each ELISA method. Despite the lack of critical thresholds for these predators, several specimens showed extremely strong positive responses from visual inspection of the assay plates (Figures 3 and 4C,D). Again, positive ELISA reactions were encountered more frequently with the anti-rabbit IgG ELISA.

Discussion

The results from these studies clearly showed that the anti-rabbit IgG sandwich ELISA was both more specific and sensitive than the *H. armigera*-specific indirect ELISA. In a previous study, a sandwich ELISA specific to pink bollworm, *Pectinophora gossypiella* (Saunders), was more effective at detecting predation by *Hippodamia convergens* Guerin-Meneville than a pink bollworm-specific indirect ELISA (Hagler, 1998). Hagler et al. (1997) suggested that the high (non-target) protein content of these predatory beetles might negatively affect the binding capacity of the targeted prey protein in the indirect ELISA format. In short, the sandwich ELISA format appears to be the more sensitive assay irrespective of the antibody used. Interestingly, the *H. armigera*-specific ELISA was least effective and the probability of detection declined more rapidly over time in the coccinellid, *H. variegata* than in the melyrid, *D. bellulus*. However, detection of predation with the rabbit IgG-specific ELISA declined more rapidly over time in *D. bellulus* than in *H. variegata*.

It appeared that some non-target proteins in the arthropod predators examined in this study cross-reacted to the *H. armigera*-specific MAb. This led to substantial background noise in the *H. armigera*-specific ELISA, thus increasing the probability of false-negative results. This demonstrates the need to conduct thorough cross-reactivity tests for each prey-specific ELISA for every predator and prey occurring in the habitat of interest. Effectiveness in one situation may not transfer to another. It should be noted that the *H. armigera*-specific MAb used in this study was not originally developed for detection of predation (Trowell et al., 2000), but to differentiate *H. armigera* from *H. punctigera* eggs. A new *H. armigera*-specific MAb developed specifically for detection of predation may yield better results, particularly if the sandwich ELISA format is used. In contrast to the *H. armigera*-specific ELISA, cross-reactivity was absent using the anti-rabbit IgG ELISA with very obvious positive results that were easy to interpret.

Longer retention of rabbit IgG within the gut contents of these two predators coupled with monospecificity of the anti-rabbit IgG make the prey marking technique a more effective method for detection of predation on *H. armigera* in the field. This is supported by the greater proportion of

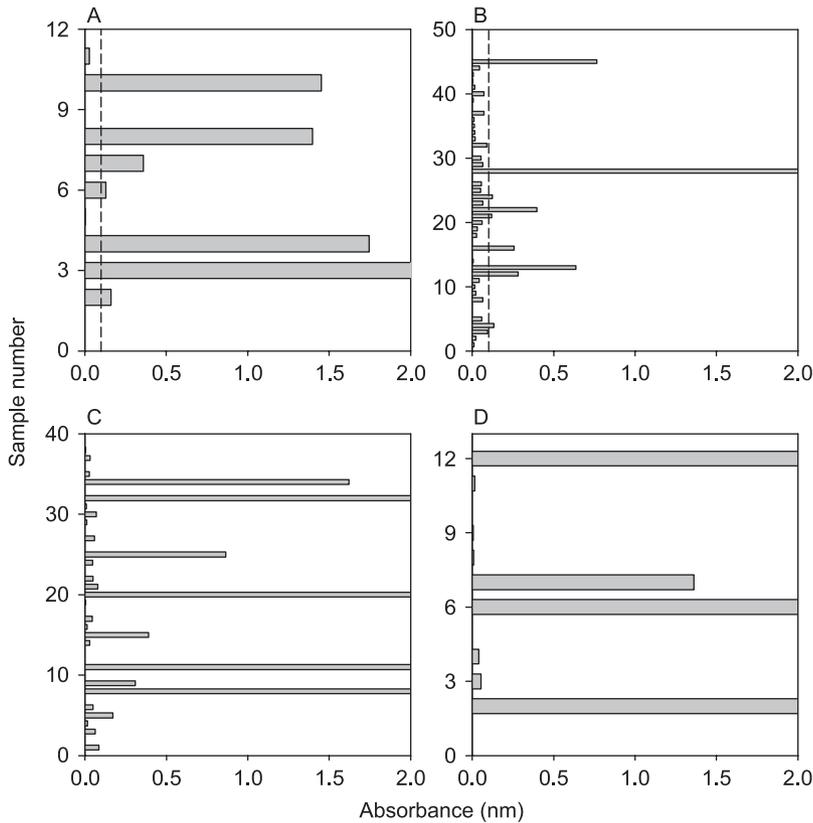


Figure 3 Absorbance values from the rabbit immunoglobulin G (IgG)-specific sandwich enzyme-linked immunosorbent assay (ELISA) for field-collected specimens of (A) *Dicranolaius bellulus*: 64% positive, $n = 11$; (B) *Hippodamia variegata*: 18% positive, $n = 45$; (C) *Coccinella transversalis*: 16% positive, $n = 38$; and (D) *Cheiracanthium spec.*: 33% positive, $n = 12$. Dashed lines indicate positive detection thresholds for *D. bellulus* and *H. variegata*.

field-collected predators that tested positive for recent predation using the rabbit IgG ELISA. Although not specifically tested in this study, there are no reports of insect predators being attracted to the rabbit IgG marker on its own (Hagler & Jackson, 2001). Positive detection of feeding by *D. bellulus*, *C. transversalis*, and *Cheiracanthium spec.* supports earlier reports that these arthropods prey upon *H. armigera* (Room, 1979; Horne et al., 2000; Scholz et al., 2000). To our knowledge, this is the first record of *H. variegata* feeding upon eggs of *H. armigera* in Australia. The impact of these predators on *H. armigera* populations cannot be determined from this field study, because it was very limited (a single event on small, early season cotton plants). Predation on the marked *H. armigera* eggs may have been greater than normal, because very few other prey were present in the crop at the time of the experiment (S Mansfield, pers. obs.).

A key factor that contributed to higher detection rates with the rabbit IgG-specific ELISA in this study was that the rabbit protein marker persists longer in the gut contents of predators than does the antigen detected by the *H. armigera*-specific MAb. The rate of antigen decay is an important limiting factor for all ELISA tests. Ideally, the chosen antigen–antibody combination will have the optimum decay rate for the intended environment. If the

target prey has a patchy distribution and variable levels of abundance, a slower decay rate may aid identification of key predators by increasing the likelihood of detection. On the other hand, if the target prey is usually widespread and highly abundant, a faster decay rate allows better quantification of predation over time (Hagler, 2006). For example, a Diptera-specific MAb with a comparatively short antigen detection period was very effective for investigation of spider feeding behaviour in alfalfa (Harwood et al., 2007).

Of the two ELISA methods, the rabbit IgG-specific sandwich ELISA was superior to the *H. armigera*-specific indirect ELISA for detecting prey in the guts of the two predator species tested. While the observed predation rates in field-collected predators suggest that *D. bellulus* and *H. variegata* may contribute to suppression of *H. armigera* populations, this needs to be confirmed by large-scale field studies throughout the growing season.

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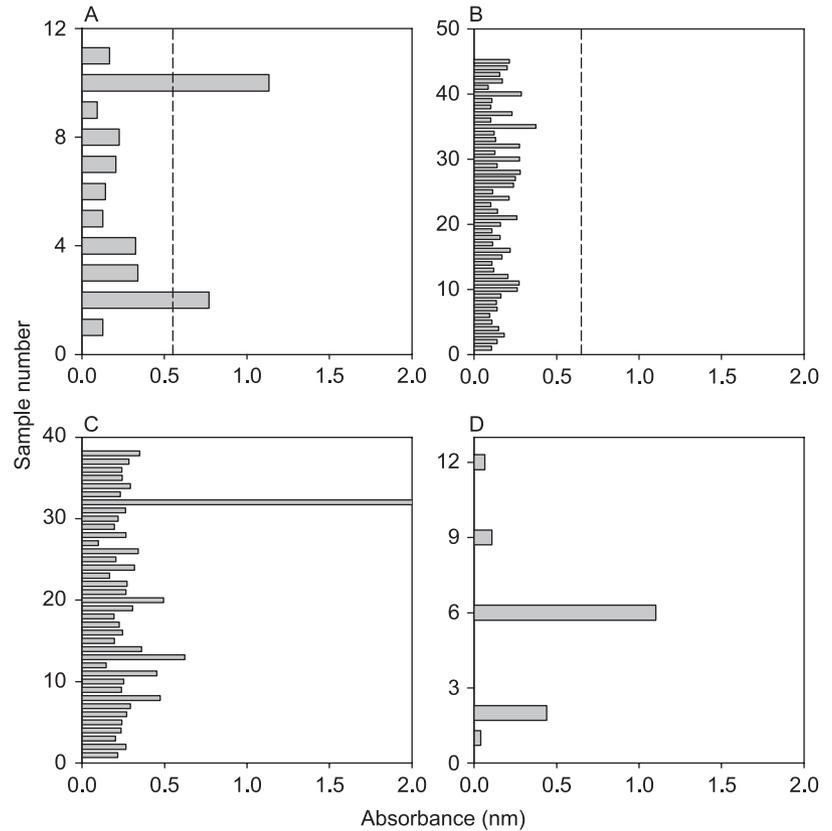


Figure 4 Absorbance values from the *Helicoverpa armigera*-specific indirect enzyme-linked immunosorbent assay (ELISA) for field-collected specimens of (A) *Dicranolaius bellulus*: 18% positive, $n = 11$; (B) *Hippodamia variegata*: 0% positive, $n = 45$; (C) *Coccinella transversalis*: 3% positive, $n = 38$; and (D) *Cheiracanthium spec.*: 8% positive, $n = 12$. Dashed lines indicate positive detection thresholds for *D. bellulus* and *H. variegata*.

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